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Research Article

New records of digenetic trematodes infecting *Melanoides tuberculata* (O.F. Müller, 1774) in Florida, USA

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Abstract

Melanoides tuberculata is a well-known invasive, freshwater snail and intermediate host for many species of parasitic trematodes. Despite being well established in Florida's freshwater systems, little work has been conducted to document the associated trematodes of the snails. Live snails were collected from 11 sites from 25 locations sampled across the state of Florida, USA of which individuals from six sites were found to be infected with trematodes. Gross morphological characters and phylogenetic analysis using 18S rRNA and ITS2 gene regions, were used to identify these trematode taxa. Snails infected with the gill flukes, *Centrocestus formosanus* and *Haplorchis pumilio* (Heterophyidae) were found at two and four sites, respectively. While *C. formosanus* has been documented in this snail species in Florida, *H. pumilio* has not been documented in Florida before this study. The eye fluke, *Philophthalmus* sp. (Philophthalmidae) was found in two sites. This parasite was previously reported in Florida in birds but not in any snail hosts. An unidentifiable species of kidney fluke, Renicolidae sp., and an unidentifiable species of bat fluke, Lecithodendriidae sp., were found in one site each. Both Renicolidae sp. and Lecithodendriidae sp. are new North American records of trematodes infecting *M. tuberculata*, but whether they are they are of native or invasive origin is unknown. These new records of trematode taxa infecting *M. tuberculata* in Florida emphasizes the critical need for monitoring of invasive snails and their associated parasites.

Key words: invasive species, freshwater, parasite, snail, barcoding, ITS2, 18S rRNA

Introduction

Melanoides tuberculata (O. F. Müller, 1774) (Caenagastropoda: Thiaridae) are aquatic snails of Asian and African origin that have achieved a global distribution in tropical and subtropical habitats and warm water systems (see citations of invasion localities and dates in Tolley-Jordan and Chadwick 2012). Although the invasion of these species has resulted in the displacement of native snails in the USA (Murray 1971), an equally alarming issue is that these invasive snails are a primary host to ~50 trematode taxa (Trematoda: Digenea) in their native range of which many cause diseases in fishes and waterfowl and some are pathogenic to humans (Pinto and Melo 2011). The transmission of trematode parasites to new aquatic systems can occur

through multiple pathways including the aquarium industry, particularly through the ornamental fish trade as documented in Mexico (Martínez-Aquino and Aguirre-Macedo. 2019), and through highly mobile definitive hosts that can move the parasite to far-reaching, novel habitats (Scholz and Salgado-Maldonado 2000).

In aquatic systems, a typical life cycle of a digenetic trematode is characterized by adult worms in or on a definitive host that release eggs that transform into miracidia. In most cases, snails are the first intermediate host where miricidia are either ingested or enter the snail through mantle cavity. Asexual reproductive sporocysts and/or rediae are formed and produce free-swimming, motile cercariae that subsequently encyst as metacercariae on substrates or in a variety of invertebrate or vertebrate second intermediate hosts. These metacercariae metamorphose into adult worms that are generally ingested and form endoparasites of aquatic or terrestrial vertebrates; albeit some adult worms metamorphose into ectoparasites (e.g. Transversotrematidae) on their definitive host (Schell 1985). Species-specific snail hosts are often required for trematodes (Sapp and Loker 2000a, b) to undergo asexual reproduction. This means that host snails are required for the establishment of invasive trematodes into new aquatic systems (see Miura et al. 2005). As such, focused surveys of invasive populations of the obligate host snails can be a strong indicator of established trematode communities or a recent invasion.

In North America, digenetic trematode infections of *M. tuberculata* have been reported throughout Mexico (Scholz and Salgado-Maldonado 2000; Scholz et al. 2001) and in the USA in Texas (Philophthalmidae: *Philophthalmus gralli*, Heterophyidae: *Haplorchis pumilio* and *Centrocestus formosanus*; Nollen and Murray 1978; Mitchell et al. 2005; Huston et al. 2014). In Florida, *C. formosanus* was documented in two locations with *M. tuberculata* in the 1980s (see Mitchell et al. 2005) and adult worms of *P. gralli* were reported in multiple bird species (Greve and Harrison 1980; Spalding et al. 1996) but have not been documented in snails. To our knowledge, there are no additional records of trematodes infecting *M. tuberculata* in Florida, despite the documented widespread distribution of *M. tuberculata* in freshwater systems (U.S. Geological Survey 2020; Wingard et al. 2008). Here, we show the necessity of conducting parasite surveys of areas with established *M. tuberculata* populations to document the invasion of parasites into Florida freshwaters.

Materials and methods

Based on published *M. tuberculata* records (Wingard et al. 2008; U.S. Geological Survey 2020), we sampled 25 waterbodies across a variety of freshwater habitats throughout Florida (Figure 1). At each location, snails were collected using dipnets or hand collection for at least 1 hour or until we collected 100 individuals. In addition, our collections were targeted for

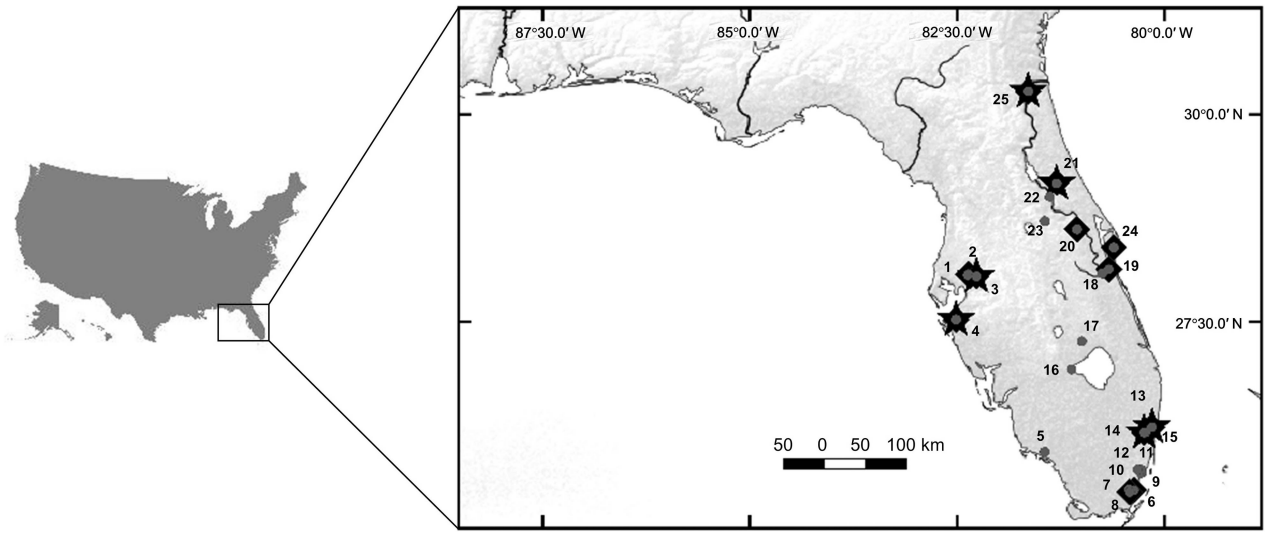

Figure 1. Locations sampled for *M. tuberculata* and their associated digenetic trematodes in Florida, USA. Sites are indicated by small grey circles and site numbers. Locations where trematode infections were found are indicated by black stars; locations where snails without trematode infections were found are indicated by black diamonds. Latitude and Longitude for each site are found in Table 2.

individual specimens > 15 mm as smaller individuals are less-likely to be infected (Tolley-Jordan and Chadwick 2019).

Tissue dissections involved the removal of a ~ 2 mm portion of gonad. This tissue was then prepared on standard glass microscope slides. Slides were viewed with a compound light microscope at 10x magnification and trematode infection was identified by the presence of immature sporocysts (early development larva stage in snails), mature sporocysts, rediae and/or cercariae. Infected tissues were photographed and gross morphological characters of cercariae were designated as morphotypes based on illustrations in Frandsen and Christensen (1984) and Schell (1985). Individual snails with confirmed parasite infections were preserved in 95% ethanol for trematode identifications using DNA extractions, sequencing, and phylogenetic analysis.

Based on Routtu et al. (2014), an 800 bp region of the 18S rDNA gene of the trematode DNA using the forward primer 5'-ATGGCTCATTAAAT CAGCTAT-3' and reverse primer 5'-TGCTTTGAGCACTCAAATTTG-3' was targeted. We selected the 18S rRNA gene because this approach has been previously validated by comparing the trematode identification infecting *M. tuberculata* using both traditional morphological and DNA-based phylogenetic analyses (Routtu et al. 2014). Additionally, the ITS2 gene was used because 18S rRNA provided equivocal results for *Haplorchis* species-level identifications. For the ITS2 analysis, a 440 bp region of the forward primer 5'-GGTACCGGTGGATCACTCGGCTCGTG-3' and the reverse primer 5'-GCTATCCTGAGGGAAACTTCG-3'was targeted (Le et al. 2017). ITS2 is a robust approach for *Halporchis* spp. identifications due to the absence of tandem repeats in *H. pumilio* and presence of tandem repeats in *H. taichui* (Le et al. 2017).

DNA extractions from snail gonadal tissue were completed using the DNeasy Blood and Tissue kit protocols (Qiagen, Valencia, CA). Each sample was centrifuged to form a pellet, and the ethanol was decanted prior to trematode DNA extraction. DNA amplifications were performed with polymerase chain reactions (PCR) carried out in 25 µL volumes using 12.5 µL of BioMix Red (Bioline, Taunton, MA), 2.5 µL (10 mM) of the forward and reverse primers described above, 5 µL of sterile deionized water, and 5µL of DNA. PCR was performed in a thermal cycler following a modified version of Routtu et al. (2014) with initiation at 94° C for 2 min followed by 30 cycles of denaturation at 94° C for 15 seconds, annealing at 60° C for 15 seconds, and elongation at 72° C for 15 seconds, and a final elongation step for 5 minutes at 72° C. Amplicons were confirmed on a 1% agarose gel stained with SYBR Green (ThermoFisher Scientific). Amplicons were cleaned using ExoSAPIT (ThermoFisher Scientific) following the manufacturer's protocol. DNA sequencing was completed at Molecular Cloning Laboratories (MCLAB; South San Francisco, CA).

Recovered sequences were assembled, verified, and aligned using the MUSCLE algorithm in Mega X (Kumar et al. 2018). Basic Local Alignment Search Tool (BLAST; Altschul et al. 1990) was used to obtain sequences with > 95% sequence coverage and match for further phylogenetic reconstruction (Table 1). Accessioned sequences of Aspidogastrea: Aspidogastridae and Digenea: Teleorchidae and Spirorchidae served as the outgroup for 18S rRNA (Olson and Tkach 2005; Table 1). For ITS2, *H. pumilio* and *H. taichui* were used as reference sequence (Table 2) and *H. yokogawi* served as the outgroup. For both genes, sequence alignment introduced gaps which were treated as binary, presence/absence characters (Giribet and Wheeler 1999).

Sequence alignments were analyzed with maximum parsimony (MP) and Bayesian inference (BI). The MP analyses were conducted using PAUP* 4.0b10 (Swofford 2003), and BI was conducted using MrBayes 3.2 (Ronquist et al. 2012). The MP analyses used the heuristic search option with 1,000 random-addition-sequence (RAS) replicates and tree bisection and reconnection (TBR) branch swapping. Strict consensus trees were calculated for all MP analyses, and branch support was estimated with 1,000 bootstrap replicates (Felsenstein 1985) using heuristic searches as described above. The BI was conducted using flat priors. In all searches, three heated chains and a single cold chain were used, and runs were initiated with random trees. Chains were run for 10 million generations, and trees were sampled every 1,000 generations. A majority-rule consensus of the remaining trees was calculated to obtain a topology and posterior probabilities (PP). The BI searches were repeated three times to confirm that searches converged on the same topology. Branch support was assessed according to a 70% bootstrap criterion for MP and a 0.95 posterior probability measure for BI (Mason-Gamer and Kellogg 1996; Wilcox et al. 2002) trematode taxa were identified to the lowest taxonomic level based on clades recovered from the phylogenetic analyses.

Table 1. Accessioned sequences from GenBank for 18S rRNA used in the phylogenetic analyses. ** indicate unpublished sequences in NCBI: GenBank.

Order	Family	Species	GenBank accession	Reference
Aspidogastrida	Aspidogastridae	*Rhodella* sp.	KC181852	Giese et al. 2012 **
	Spirorchidae	*Vasotrema* sp.	AY604714	Snyder 2004
Diplostomida	Diplostomatidae	*Posthodisplostomum* sp.	KY809062	Lovy and Friend 2017
Plagiorchiida		Philophthalmidae sp.	AJ287560	Cribb et al. 2001
		Philophthalmidae sp.	AY222133	Olson et al. 2005
		Philophthalmus gralli	JX121229	Literák et al. 2013
		Philophthalmidae sp.	MT568770	***This study***
			MT568771	
	Heterophyidae	*Centrocestus formosanus*	AY245759	Flowers et al. 2004**
		Centrocestus formosanus	HQ874608	Thaenkham et al. 2011**
		Centrocestus formosanus	MT551512	***This study***
			MT551513	
		Haplorchis pumilio	HM004195	Thaenkham et al. 2010
			HM004196	
			HM004197	
		Haplorchis taichui	KX815125	Le et al. 2017
		Haplorchis spp.	MT568775	***This study***
			MT568776	
			MT568777	
			MT568778	
			MT568779	
			MT568780	
			MT568781	
			MT568782	
			MT568783	
			MT568784	
			MT568785	
			MT568786	
	Renicolidae	*Renicola lari*	KU563698	Heneberg et al. 2016
		Renicolidae sp.	MT568774	***This study***
	Unknown	Renicolidae sp.	AY245704	Dzikowski et al. 2004
	Lecithodendriidae	Lecithodendriidae sp.	EU019964	Gibson and Rikihisa 2008
			EU019971	
			EU019974	
			EU019975	
			MT568772	***This study***
			MT568773	
			JX467570	Ciparis et al. 2013
		Lecithodendrium sp.	AY222147	Olson et al. 2005
		Paralecithodendrium sp.	AY222148	

Results

Snails were found at 11 of the 25 surveyed sites (Figure 1, Table 3). We mostly collected less than 50 individuals > 15 mm per site with no individuals > 15 mm being found at sites 1,6, and 24 (Figure 1, Table 3). In total, 264 snails from 8 sites were dissected for trematode detections.

In total, 36 individuals were found to have evidence of trematode infection. These snails were found at six sites (3, 4, 13, 14, 15, 25; Table 3). Sites 8 and 19 had no infected snails. All infected snails presented with sporocysts and/or rediae. Additionally, cercariae were generally present except for an individual collected at site 4. This snail only presented with immature sporocysts in very early development stages. However, the parasites from this snail were not included in genetic analyses. No dual infections were found in any of the infected snails.

Table 2. Accessioned sequences from GenBank for ITS2 used in the phylogenetic analyses. ** indicate unpublished sequences in NCBI: GenBank.

Order	Family	Species	GenBank accession numbers	Reference
Plagiorchiida	Heterophyidae	*Haplorchis pumilio*	HM004161 HM004162 HM004163	Thaenkham et al. 2010
		Haplorchis pumilio	GU244513 GU244514 GU244517 GU244524	Thuy et al. 2010
		Haplorchis pumilio	JX815125	Nissen et al. 2013
		Haplorchis pumilio	KP165439	Li et al. 2014**
		Haplorchis pumilio	MT102549 MT102550 MT102551 MT102552 MT102553 MT102554 MT102555 MT102556 MT102557 MT102558 MT102559 MT102560	***This study***
		Haplorchis sp.	GU244519	Thuy et al. 2010
		Haplorchis taichui	HM004155 HM004156 HM004157	Thaenkham et al. 2010
		Haplorchis taichui	KX815126	Le et al. 2017
		Haplorchis taichui	EU826640	Skov et al. 2008
		Haplorchis taichui	GU244515	Thuy et al. 2010
		Haplorchis yokagawai	HM004158 HM004159 HM004160	Thaenkham et al. 2010

Table 3. Site locations, number of *M. tuberculata* collected and number of snails dissected. Sites where snails were collected are in bold.

Site	Latitude	Longitude	# of snails collected	# of snails dissected
1	**28.0744**	**–82.3664**	**6**	**0**
2	28.0631	–82.3739	0	–
3	**28.0487**	**–82.2685**	**> 100**	**22**
4	**27.5273**	**–82.2513**	**> 100**	**48**
5	25.9314	–81.4441	0	–
6	**25.4705**	**–80.3715**	**3**	**0**
7	25.4782	–80.4287	0	–
8	**25.4485**	**–80.4199**	**> 100**	**50**
9	25.6803	–80.2728	0	–
10	25.6747	–80.2842	0	–
11	25.7180	–80.2806	0	–
12	25.7195	–80.3263	0	–
13	**26.2309**	**–80.1522**	**29**	**19**
14	26.1664	–80.2308	0	–
15	**26.1645**	**–80.2449**	**> 100**	**25**
16	26.9275	–80.1212	0	–
17	27.2664	–80.9963	0	–
18	28.0839	–80.7519	0	–
19	**28.1346**	**–80.6714**	**> 100**	**50**
20	28.6159	–80.0562	0	–
21	**29.1670**	**–80.3004**	**> 100**	**25**
22	29.0090	–80.3820	0	–
23	28.7139	–80.4436	0	–
24	**28.3967**	**–80.6114**	**2**	**0**
25	**30.2784**	**–80.6347**	**100+**	**25**

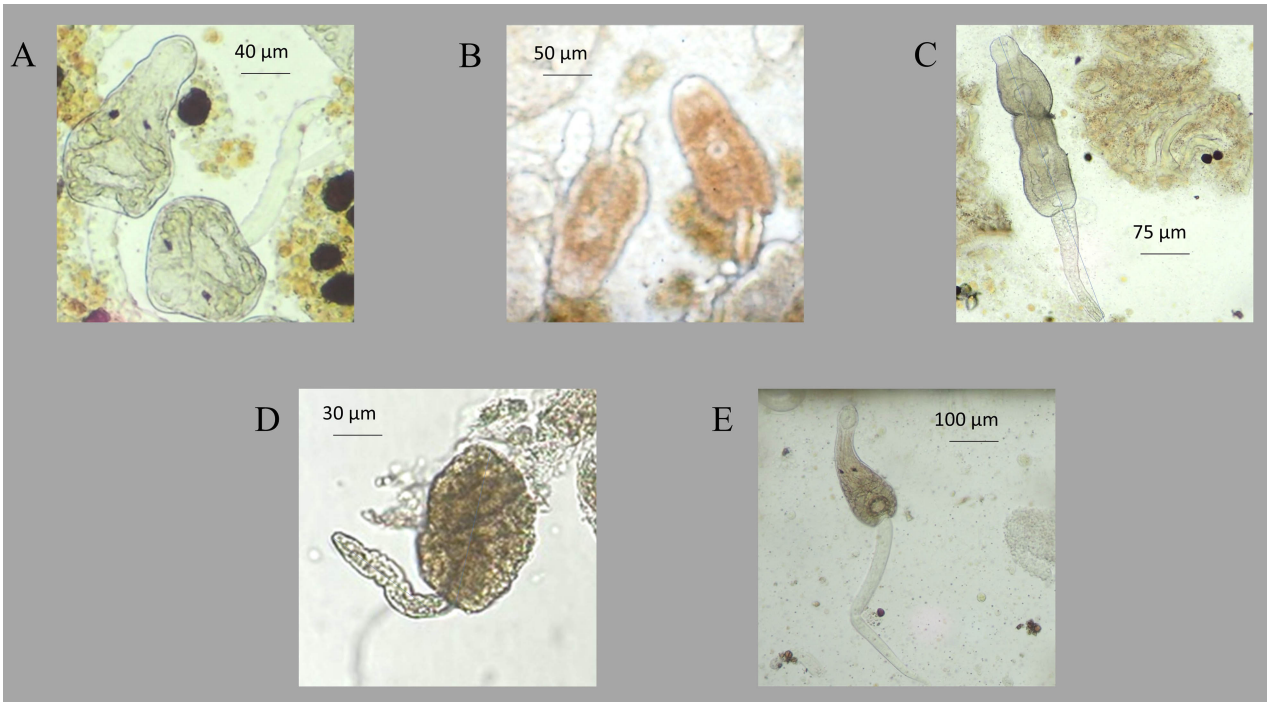

Figure 2. Photos of cercarial morphospecies observed in tissue smears. A) Heterophyidae; *Centrocestus formosanus*, B) Renicolidae sp., C) Philophthalmidae: *Philophthalmus* sp., D) Lecithodendriidae sp. and E) Heterophyidae: *Haplorchis pumilio*. Site locations for morphospecies are reported in Table 3. Confirmation of taxa identifications are based on phylogenetic analyses of 18S rDNA (A–D; Figure 3) and ITS2 (E; Figure 4) phylogenetic trees.

We identified 5 distinct trematode morphotypes in infected snails (Figure 2). Based on gross morphological characters of cercariae from Frandsen and Christensen (1984) and Schell (1985), these morphotypes matched with Heterophyidae (nominally *Haplorchis* sp. and *Centrocestus formosanus*), Philophthalmidae, Renicolidae, and Lecithodendriidae families. In addition, comparisons of published cercarial photographs of *C. formosanus*, and *H. pumilio* to photos of specimens from this study corroborate our findings. For instance, *Centrocestus* cercariae collected from snail tissues in this study (Figure 2a) had flattened v-shaped bladders, and straight tails (Krailas et al. 2014) as compared to a less distinct, round bladder and a more pronounced fin-fold in the tail of a *C. caninus* cercaria (Dunghungzin and Chontananart 2020). *Haplorchis* cercariae collected from snail tissues in this study (Figure 2e) had tail fin-fold features that more closely resembled *H. pumilio* as compared to *H. taichui* (Krailas et al. 2014).

Twenty trematode sequences were included in the 18S rRNA phylogenetic reconstruction. A data matrix consisting of 761 aligned positions plus 5 binary-coded indels was produced. A total of 170 characters were parsimony informative. Significant statistical support values recovered from both bayesian and parsimony analyses (Figure 3) resulted in all sequences being recovered in the order Plagorchiida including five trematode taxa: Heterophyidae: *Haplorchis* spp., Heterophyidae: *Centrocestus formosanus*, Philophthalmidae: *Philophthalmus sp*, Lecithodendriidae sp. and Renicolidae sp. (Figure 3, Table 4).

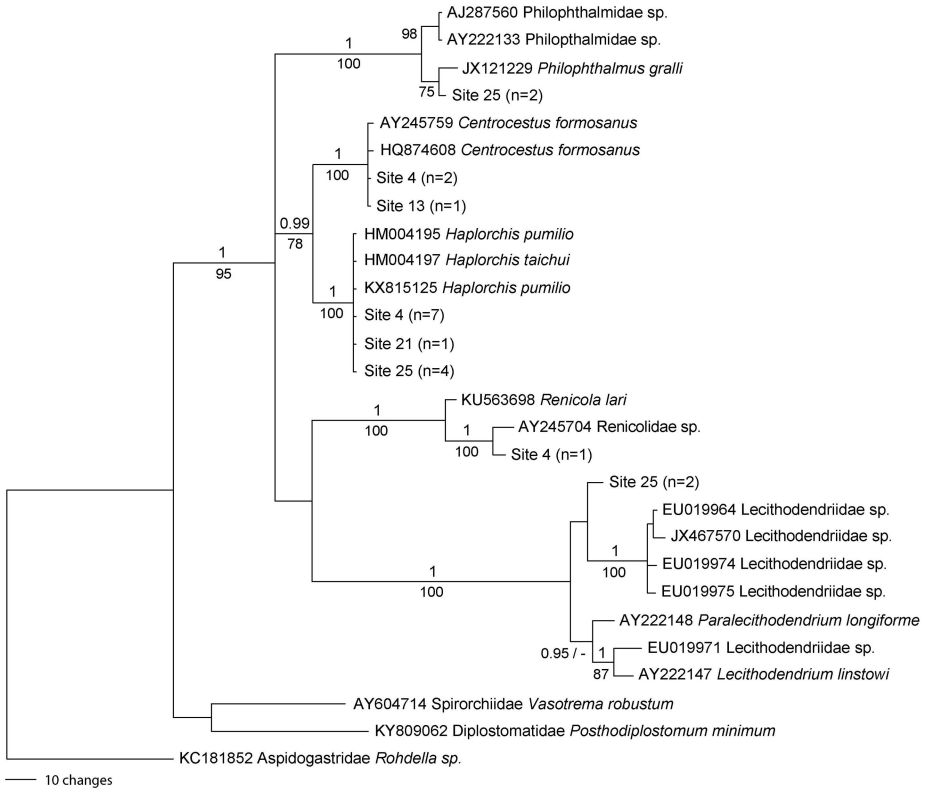

Figure 3. Phylogenetic tree obtained through Bayesian inference for the 18S rRNA dataset. Significant statistical support values recovered from both Bayesian and Parsimony analyses are included. The scale bar below represents number of nucleotide substitutions per site. In the tree, sample locations are indicated by sites with number of sequences used reported parenthetically.

Table 4. Trematode identifications by site locations. Identifications are from DNA-based phylogenetic analysis, except for sites 3 and 15 where visual evaluation of gross morphology were used (Figure 2). Number indicates the number of dissected snails found with trematodes.

Site	A: Heterophyidae: *Centrocestus formosanus*	B: Renicolidae: Unknown sp.	C: Philophthalmidae: *Philopthalmus* sp.	D: Lecithodendriidae: Unknown sp.	E: Heterophyidae: *Haplorchis pumilio*
3	–	–	–	–	1
4	1	3	–	–	18
13	1	–	–	–	–
15	–	–	1	–	–
21	–	–	–	–	1
25	–	–	2	2	6

For *Haplorchis* spp. (i.e., *H. pumilio* and *H. taichui*), the 18S rDNA results were equivocal. Therefore, we evaluated this genus using phylogenetic reconstruction with the ITS2 gene. Twelve trematode sequences from our study sites were used for this analysis. A total of 258 characters were parsimony informative. Significant statistical support values from both bayesian and parsimony analyses confirmed the presence of *H. pumilio* only rather than both *Haplorchis* species identified in the 18S rRNA phylogenetic tree (Figure 4).

Unfortunately, DNA extracted from sites 3 and 15 were not successfully sequenced, but identification based on gross morphology suggests these parasites are likely *H. pumilio* (site 3) and an undetermined species of *Philophthalmus* sp. (site 15).

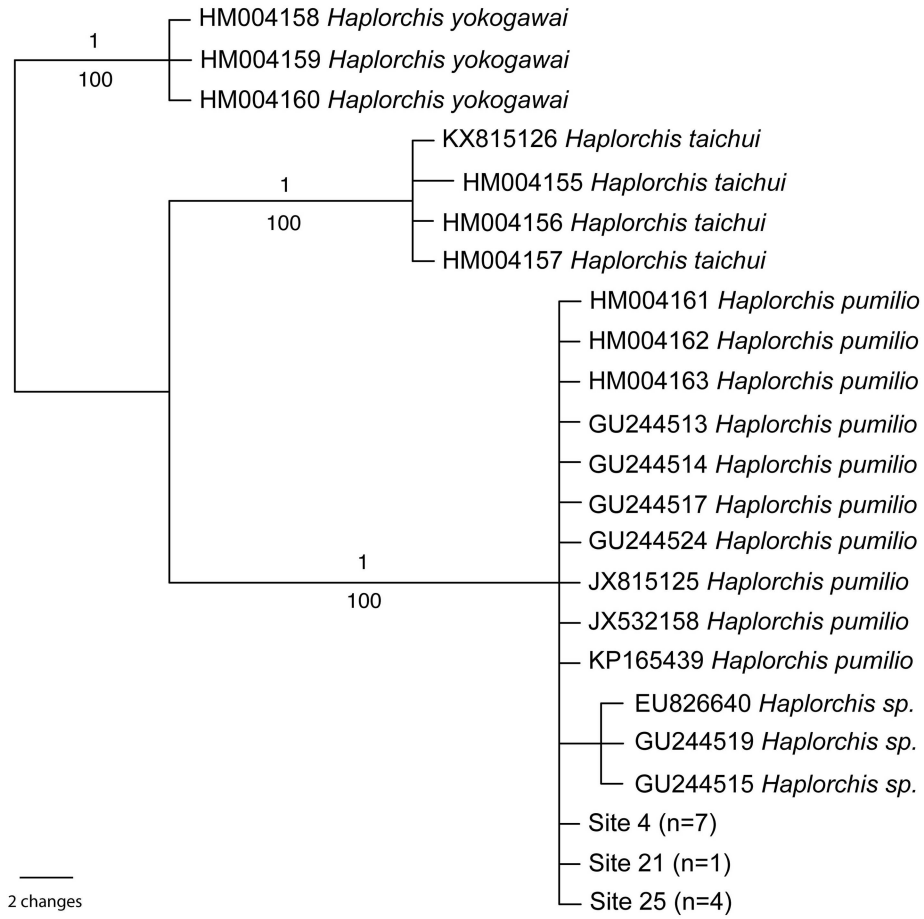

Figure 4. Phylogenetic tree obtained through Bayesian inference for the ITS2 region of rRNA dataset. Significant statistical support values recovered from both Bayesian and Parsimony analyses are included. The scale bar below represents number of nucleotide substitutions per site. Only the presence of *Haplorchis pumilio* in our samples is supported by this analysis.

Discussion

In this study we have documented several taxa of digenean trematodes infecting of the invasive snail *M. tuberculata* Using a combination of phylogenetic analysis and gross morphological characters of cercariae proved effective in finding two heterophyid taxa (*Centrocestus formosanus* and *Haplorchis pumilio*, an eye fluke (Philophthalmidae: *Philophthalmus sp.*), a kidney fluke (Renicolidae sp.) and a trematode taxon previous only associated with bats in North America (Lecithodendriidae sp. – Schell 1985).

Centrocestus formosanus, *H. pumilio* and *H. taichui* are all are known to cause heterophyiasis in humans in Southeast Asia, but the greatest concern of these parasites in the USA is fish health. As these parasites encyst in fishes in different locations (gills for *C. formosanus* (Martin 1958), fin insertions for *H. pumilio* (Huston et al. 2014), and at the base of the tail for *H. taichui* (Martin 1958). The statistical support of the ITS2 analysis that placed Florida trematode taxa into *H. pumilio* suggests that other species of *Haplorchis* (*H. taichui* and *H. yokogawi*) have not invaded sites sampled in the study, but more robust sampling is needed to verify this finding.

Although other invasive taxa of Heterophyids were not recovered at these sites, these taxa could be in other snail populations in the state. It is worth noting that two GenBank accessions published as *H. taichui* (GU244515 – Thuy et al. 2010 and EU826640 – Skov et al. 2008) were recovered as *H. pumilio* in our analysis based on the absence of tandem repeats in ITS2 (Le et al. 2017). Based on these results, comparisons of cercarial morphology of taxa collected by Skov et al. (2008) and Thuy et al. (2010) to our study is needed. Given the findings of *H. pumilio* at multiple sites throughout the state, monitoring of snail populations in springs or other habitats with endemic and/or imperiled fishes is necessary for effective conservation strategies. In addition, owners of ornamental fish hatcheries that draw water from systems that harbor infected snails should be alerted as that are not able to sell fishes infected with these parasites (Mitchell et al. 2005).

Currently, three avian eye flukes are known to use *M. tuberculata* as a first intermediate host. These include: *P. nocturnus* (Madhavi et al. 1997) reported in Egypt, *P. distomatosa* in Israel (Radev et al. 2000), and *P. gralli* that is native to the Middle East but has established populations infecting *M. tuberculata* in North and South America (Pinto and Melo 2011). All other records of *Philophthalmus* species from North America are in birds (Florida-Spalding et al. 1996; Texas-Dronen and Blend 2008), and one report in a human eye in Mexico (Lamothe-Argumedo et al. 2003). We cannot confirm the species of *Philophthalmus* that we recovered given the lack of available 18s rRNA sequences that can be aligned with samples collected in our study. Adult worms of this species infect the nictitating membranes which may lead to blindness in birds (Nollen and Murray 1978), therefore endangered, threatened, or migratory bird populations that co-occur with these snails should be closely monitored.

No published reports of Lecithodendriidae infecting *M. tuberculata* in North America were reported prior to this study, as far as we are aware. Thus far, only *Lecithodendrium pyradium* (Abdel-Azim 1936) and *Pleurogenoides* spp. (Yousif et al. 2010) reported infecting *M. tuberculata* from Egypt, and *Loxegenoides bicolor* infecting *M. tuberculata* in Thailand (Krailas et al. 2014) are published records of Lecithodendriidae infecting *M. tuberculata*. The generalized life cycle for this family includes freshwater operculate snails as the first intermediate hosts where virgulate cercariae emerge and encyst as metacercariae in various aquatic, larval insects and adult worms are considered intestinal parasites of bats (Schell 1985). Yet, this generalization should be revised as records of amphibians, reptiles, and mammals with intestinal infections of adult worms have been observed. Adult Tree frogs *Lithobates* (*Rana*) spp. were reported as definitive hosts in California (Goodman 1989), Mexico (Cabrera-Guzmán et al. 2010) and Pakistan (Coil and Kuntz 1960). *Pleurogenoides tenure* was recovered in a lizard during an experimental infection in Egypt (Macy

1964) as well as mammals. Adult worms of *Allasogonoporus* sp. were found in a muskrat in the USA (Olivier 1938), and multiple genera (*Paralecithodendriium* spp., *Prosthodendriium* spp., and *Phaneropsolus* spp.) were recorded in humans in Southeast Asia (Chai et al. 2009; Kaewkes et al. 1991). As adult worms are found in a wide variety of vertebrate hosts, determination of the lifecycle of the species found in *M. tuberculata* in Florida warrants a more detailed study using morphology and additional molecular markers. More importantly, determining if these parasites are of native origin or are invasive and potential pathogens of definitive hosts emphasizes the need to monitor invasive snail populations to detect parasite invasions.

As with the findings of lecithodendriids infecting *M. tuberculata*, no published reports of Renicolidae spp. infecting these snails in North America were reported prior to this study. A review of the taxonomic literature by Stunkard (1964) for these taxa showed that this family has a broad distribution across the globe in tropical, sub-tropical, and temperate biomes; albeit, first intermediate hosts are listed as marine snails. An extensive, multi-year study on the east coast of the US showed marine snails as the first intermediate host, clams as the second intermediate host, and a variety of birds as the definitive hosts (see Stunkard 1964) while Martin (1971) found *Renicola* spp. found on the west coast of the USA used marine, cerithioid snails as first intermediate hosts, killifish served as the intermediate host and marine birds as the definitive hosts. Interestingly, Sharp (2008) conducted a study in Florida on trematodes found in native, freshwater apple snails (*Pomacea paludosa*) and found one unnamed species of Renicolidae with morphology and molecules (18S rDNA gene) but the genetic data from this study has not been made available on GenBank for comparisons to our study. Pinto and Melo (2012) reported a putative *Renicola* sp. infecting *M. tuberculata* in Brazil and alluded to the invasive origin of this species while Krailas et al. (2014) reported a species of *Renicola* infecting these snails in Thailand. However, only morphological characters were used in these studies. Despite the inconclusive identification of this species, the fact that these are kidney parasites could lead to impaired health of vertebrate intermediate and definitive hosts.

We primarily relied on 18S rRNA as the genetic marker for determining taxonomic identifications of these trematode parasites. While this is a very effective, reliable marker in identifying these parasites, other gene regions of ribosomal rRNA, such as 28S rRNA, have more accessioned sequences which could improve overall detection across this group (Pérez-Ponce de León and Hernández-Mena 2019). As a further example, our addition of ITS2 did allow us to confirm the absence of *H. taichui* from our samples which was suggested to occur in our study areas by the 18S rRNA analysis. Ultimately, using multiple gene markers will better elucidate identities of

larval trematodes, particularly in studies aiming to determining if trematodes are of native or invasive. However, despite the utility of multiple gene markers, there is a major gap in the reference sequences available for trematodes that are not medically relevant (Poulin et al. 2019). Regardless, our approach did allow us to confirm the presence of 5 trematode taxa parasitizing *M. tuberculata* at our study locations.

Trematode identifications based on genetic analyses, no matter how coarse in resolution, clearly provide parasitologists with important information to support investigations relating to biodiversity, life-cycle information, and pathogenicity to human or wildlife health. While we recognize that the utility of molecular markers for the identification of trematodes in infected snails is not substitute for detailed morphological studies on larval stages, our approach can be useful when conducting geographically, wide-spread surveys within a limited timeframe. For example, in our study, processing of shed cercariae or staining specimens of larval trematodes separated from snail tissues would not have been possible given the time constraints in this study. As such, confirming larval parasites in tissue smears and recorded generalized cercarial forms allowed us to selected samples to further process for genetic analysis. Unfortunately, diagnostic characters needed for identification are often not visible in early larval developmental stages (such as immature sporocysts) nor in cercariae damaged from creating tissue smears. Therefore, established methods for identifying trematodes based on shed cercariae (i.e., Schell 1985), would not provide definitive identifications. However, comparisons of trematode gross morphology from images associated with snail tissue smear in combination with genetic analysis was successful in identifying some trematodes to species and others to at least the family level.

The objectives of this study were to determine the geographic distribution and identification of trematodes infecting *M. tuberculata* populations in Florida. Our results showed a widespread distribution of infected snails that included multiple trematode taxa at some locations, all of which, except *C. formosanus*, are new records for *M. tuberculata* in Florida. As the dispersal of trematodes to new waterways occurs via direct movement of infected snails and/or indirect transmission via secondary and definitive hosts (Scholz and Salgado-Maldonado 2000), this widespread distribution was not an unexpected finding. As our study covered only a small percentage of the waterways in Florida, these results are clearly a conservative estimate of trematode taxonomic richness, parasite prevalence, and distribution of infected snails in Florida. In addition to highlighting the need for sampling *M. tuberculata* populations to document the invasion of trematode species, this study also demonstrates the need future, detailed studies of life cycles of poorly described taxa to evaluate the potential health concerns to wildlife.

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Authors' contribution

L. Tolley-Jordan and M. Chadwick equally contributed for the research conceptualization, sampling design and methodology, investigation and data collection. J. Triplett analyzed genetic data. LTJ wrote the initial draft and LTJ and MAC reviewed and edited the manuscript. No conflict of interest influenced the data provided in this manuscript.

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